Growth of the Early Chick Thyroid and Its Relationship to Thyroid Morphogenesis[1]

GWENDOLYN M. KINEBREW AND SARAH MACFARLAND, Department of Biology, John Carroll University, University Heights, OH 44118

ABSTRACT. **We used both qualitative and quantitative techniques to test the hypothesis that lateral expansion of the developing chick thyroid is restricted structurally. To do this, we isolated pharynxes from embryos of stage 13 to stage 15, the period during which evagination is occurring, and measured the amount of pharyngeal floor area occupied by thyroid, using the raised ridge at its periphery to define its limits. These measurements were then compared with volumetric ones of the same thyroids. Additionally, living isolated pharynxes were treated with dihydrocytochalasin B, a compound known to disrupt actin filaments. The results showed that growth of the thyroid (as indicated by its volume) is not accompanied by expansion of the primordium into the surrounding pharyngeal space (as indicated by its area). In addition, treatment with dihydrocytochalasin B caused flattening and spreading of the raised ridge of cells that bounds the thyroid pit suggesting that microfilaments are involved in maintenance of this structure. A discussion of the results in relation to previously reported findings implicates microfilaments in both the formation and maintenance of the peripheral cell ridge.**

OHIO J SCI 101 (3/4):42–47, 2001

INTRODUCTION

The morphogenetic events that result in the formation of an organ do not occur in isolation, but rather are influenced by the local environment (Hilfer 1973; Hardin 1990). If we are to increase our understanding of how 3-dimensional structure is generated, we must identify those components of the system in question that can impact final organ shape. One method by which this can be determined is the quantitative description of structure (Koehl 1990) coupled with qualitative experimentation. The present study uses such an analysis to study chick thyroid morphogenesis, a system in which little recent work has been done.

The descriptive morphology of the developing chick thyroid has been reported (Hopkins 1935; Shain and others 1972; Hilfer 1973). The thyroid develops along the midline of the ventral floor of the pharynx at the level of the second visceral arch. The gland is first evident at stage 12 (Hamburger and Hamilton 1951) when it is distinguishable from surrounding tissue by the greater amount of apical blebbing and the closer apposition of its cells. At stage 13 the thyroid is a shallow depression surrounded by a raised ring of cells at its periphery. At stage 14 and continuing into stage 15, the thyroid appears as concentric rings of cells with the outermost cell ring elevated above the level of the pharyngeal floor. Longitudinal sections reveal that the thyroid is now a cup-shaped pit with a cranial bias. Beginning at stage 15, the opening to the thyroid becomes progressively restricted until it is completely closed off from the pharynx at stage 21. As seen in many other organs of epithelial origin, the cells of the thyroid placode contain thick bands of microfilaments just below the apical and basal surfaces (Shain and others 1972; Hilfer 1973). Longitudinally oriented bands of microfilaments also are present in cells that overlie grooves in the basal surface of the organ (Hilfer 1973). Based on these observations, Hilfer (1973) proposed that evagination of the thyroid may occur as a result of bending in response to forces exerted by surrounding tissues, while apical and longitudinal bands of microfilaments may act to stabilize organ structure. The fact that this system can evaginate precociously in response to an ATP-containing medium (Hilfer and others 1977) implicates contractile forces in thyroid evagination and suggests an active role for microfilaments (Lewis 1955; Wessells and others 1971; Burnside 1973; Schroeder 1973) in the morphogenesis of this system.

We focused on a single morphological feature of early thyroid organogenesis, the ridge of cells that surrounds the evaginating primordium. We asked whether this ring of cells plays a role in limiting the lateral expansion of the thyroid. A two-pronged approach was used to address this question, one quantitative and one qualitative. Pharynxes excised from embryos of stages 13 to 15 were used to quantitate the morphological changes in the thyroid during this time period. We measured the amount of surface area occupied by the thyroid within the floor of the pharynx and also determined the volume of the thyroid pit (which results from thyroid evagination) from stage 13 to stage 15. In addition, living pharynxes, maintained in warm buffered saline, were exposed to dihydrocytochalasin B. Dihydrocytochalasin B causes depolymerization of actin filaments by capping the fast growing end of the filaments. (Mabuchi 1983; Bonder and Mooseker 1986). The results of this study corroborate the preliminary findings of Hilfer (1973) that growth of the thyroid is accompanied by constraints on its lateral expansion into pharyngeal space and implicate microfilaments in stabilization of the peripheral cell ridge that defines the limits of the early thyroid.

[1]Manuscript received 7 February 2000 and in revised form 3 August 2000 (#00-01).

MATERIALS AND METHODS

Culture and Microscopy of Living Preparations

White Leghorn chicken eggs (Truslow Farms, Chestertown, MD) were incubated at 39° C in a Quincy Labs, Inc. (Chicago, IL) model 12-140 incubator for 56 to 65 hours to obtain embryos of stages 13 to 15 of development. All staging was according to the criteria of Hamburger and Hamilton (1951). Intact embryos were removed from the yolk and placed in 100 × 20 mm tissue culture dishes in phosphate buffered saline (PBS) or Ringer's isotonic chick saline. The extraembryonic membranes were removed and the pharynx was isolated by removal of tissue caudal to the heart and cranial to the mandibular arches, followed by excision of the heart and neural tube. The pharyngeal roof and any neural tissue attached to the pharyngeal arches that might obscure the view of the primordium were also removed. The isolated ventral pharynx was transferred to a depression slide containing fresh saline and positioned lumen side up.

Measurements and Calculation of Area and Volume

To determine the depth of the thyroid, each specimen was placed on the stage of an Olympus model CH-2 microscope. Working at 100× magnification, the microscope was initially focused at the level of cell apices immediately adjacent to the pharyngeal floor. This region of cells forms a raised ridge surrounding the thyroid pit. After noting the lens position, the microscope was refocused on the deepest discernible point of the thyroid pit; a scale on the fine focus adjustment knob (2.5 μm per tick mark) was used to determine the refocus distance in microns. The pharynxes were then fixed overnight in neutral buffered formalin, washed with Coleman's wash, and embedded in paraffin.

Digitized photographic images of fixed pharynxes were used to measure the cranial to caudal and lateral limits of the thyroid pit. Using Image Pro Plus, version 2.0 (Media Cybernetics, Silver Spring, MD), these measurements were taken within the inner boundaries of the raised outer ring of thyroid cells. These measurements were used to calculate the surface area occupied by the thyroid and were combined with the depth measurements to calculate the volume of the thyroid pit. The area was calculated as $\pi(a/2)(b/2)$, where a = cranial to caudal diameter and b = lateral to lateral diameter. The shape of the pit formed by evagination of the thyroid is approximately elliptical and volume was therefore calculated as (1/3A)d, where A = area and d = depth. SPSS software (version 8.0, Chicago, IL) was used to perform ANOVA (LSD) and descriptive statistics. Measurement data was obtained from the thyroids of chick embryos as follows: 6 stage 13 embryos, 9 stage 14 embryos, and 4 stage 15 embryos.

Dihydrocytochalasin B Treatment

In order to minimize tissue damage while still allowing the dihydrocytochalasin B (Sigma, St. Louis, MO) to enter the cells, a stock solution of 100 μg/ml of dihydrocytochalasin B (dihydroCB) in PBS containing 0.01 units/ml streptolysin O (Sigma) was prepared. Streptolysin O permeabilizes membranes to permit cellular uptake of large or charged molecules (Bhakdi and others 1984; Hugo and others 1986). To determine microfilament involvement in maintenance of the raised ridge of cells at thyroid periphery, isolated pharynxes were kept alive in 37° C phosphate buffered saline and treated with the dihydroCB-streptolysin O solution, dihydroCB, or streptolysin O. The pharynxes were treated for a period of 20 minutes. The treatment solution was washed off with PBS or Hanks' balanced salts and the pharynxes were allowed to recuperate. Full recuperation from the dihydroCB-streptolysin O requires a period of about 2 hours. Eighteen embryonic pharynxes from embryos ranging from stage 12 to 18 of development were used in this part of the experiment.

Photography

All photographs were taken with an Olympus model SC35 SLR camera fitted to either an Olympus model SZ40 stereomicroscope or an Olympus model CH-2 microscope fitted with phase and brightfield optics. All images used for area and volume measurements were taken with Dale 9100 color film and developed and digitized by Dale Laboratories (Hollywood, FL). All black-and-white photographs were taken with Kodak TMax-100 film and developed in Kodak TMax developer (Eastman Kodak, Rochester, NY).

RESULTS

To determine the nature of the morphological changes that occur during thyroid development in the chick embryo, we measured the length, width, and depth of the evaginated region (the thyroid pit) from chick embryos of stages 13, 14, and 15 of development (Table 1, Fig. 1). We used the raised ridge of cells surrounding the thyroid pit as a marker for its lateral margins (arrows, Fig. 1d). These measurements were used to calculate the surface occupied by the thyroid as well as the volume of the thyroid pit (Table 1). Analysis of variance revealed a significant increase in the volume of the thyroid pit between stage 13 and stage 15 ($F\ 6.797 = 0.002$, $p < 0.05$)

TABLE 1

Comparison of mean measurements of thyroid primordia from stage 13, 14, and 15 chick embryos.

Stage	*n*	Diameter Cranial/Caudal (μm)	Diameter Lateral/Lateral (μm)	Depth (μm)	Area* (μm^2)	Volume** (μm^3)
13	6	45.17	40.17	38.33	1398.93	18002.97
14	9	51.00	44.00	42.89	1812.96	25627.07
15	4	57.75	43.50	58.75	1978.61	39185.12

*Area = π[(a/2)(b/2)], where a = cranial/caudal diameter and b = lateral/lateral diameter.

**Volume = (1/3A)d, where A = area and d = depth.

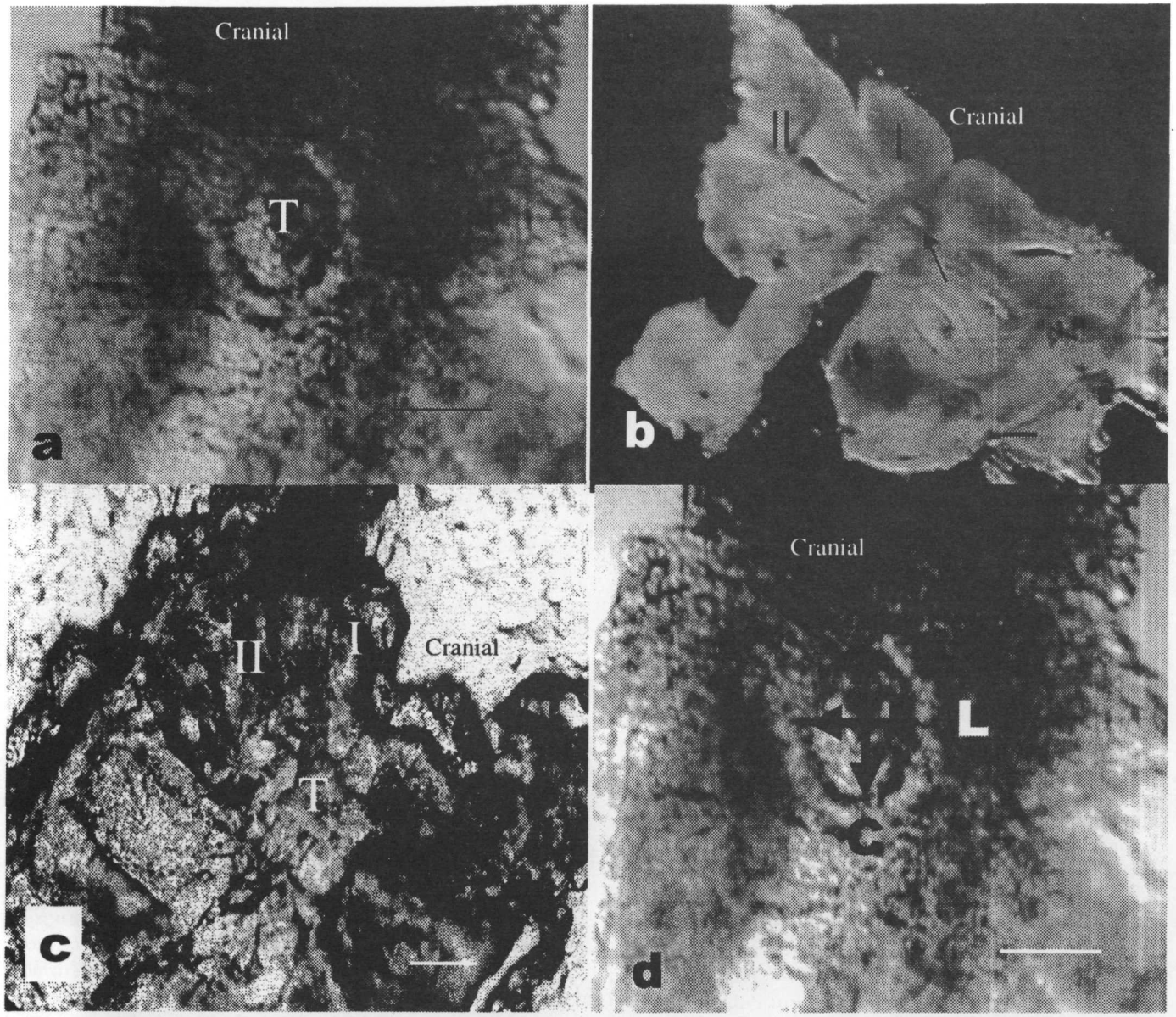

FIGURE 1. Pharynxes from stage 13, 14, and 15 chick embryos. a) Pharynx from a stage 13 chick embryo. The thyroid is indicated by the letter T and forms a slight depression at the midline of the pharynx. b) Pharynx from a stage 14 chick embryo. Arrow indicates location of the thyroid. c) Pharynx from stage 15 chick embryo. T indicates the location of the thyroid. The pharynxes in a and b were photographed cultured in warm saline, while c shows a pharynx fixed as described in the text. d) The double headed arrows show the parameters of the lateral (L) and cranial-caudal (C) measurements used in this study. Bar = 40 μm

and between stages 14 and 15 ($F\ 6.797 = 0.022$, $p < 0.05$) with no significant increase in surface area during this time period (Fig. 2). The amount of surface area within the pharyngeal floor occupied by the thyroid remains relatively constant while its volume increases during development (Table 1, Fig. 2).

Neither dihydrocytochalasin B (dihydroCB) in PBS alone at concentrations ranging from 10 μg/ml to 25 μg/ml (Fig. 3 a,b) nor 0.1 and 0.01 units/ml of streptolysin O (SLO) alone (Fig. 3 c,d) had a discernible effect on the thyroid. DihydroCB + SLO solutions ranging from 10 μg/ml to 25 μg/ml in dihydroCB concentration were tested for the ability to cause a reversible loss of thyroid morphology. The minimum effective concentration was determined to be 15 μg/ml (Table 2). Living pharynxes were treated with 50 μl of dihydroCB + SLO at 15 μg/ml dihydroCB concentration and photographed at 5-minute intervals over a 20-minute time period to document changes in thyroid morphology. The peripheral cell ridge of treated thyroids was observed to flatten and spread (Fig. 3 e,f,g) thereby widening the mouth of the forming thyroid vesicle. This result was observed for thyroids of stages 13 to 15, the period when evagination is occurring and to a lesser extent in more mature thyroids of stages 16, 17, and 18, the period when the mouth of the primordium is closing (Table 2).

DISCUSSION

This study shows that during the period of development from stages 13 to 15, the amount of pharyngeal floor area occupied by the thyroid remains relatively

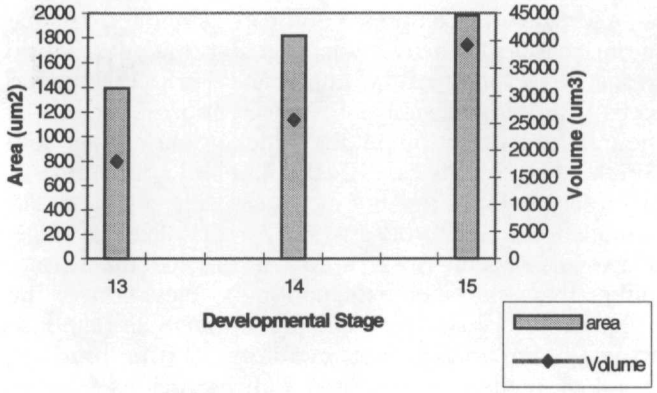

FIGURE 2. Average change in area and volume of chick thyroid during stage 13 to stage 15 of development. The volume of the thyroid and the area of the pharyngeal floor it occupies were calculated as described in the text. The graph shows the average volume (♦) and area (bars) of several thyroids for each developmental stage indicated and are based on the data summarized in Table 1. Area is the amount of pharyngeal floor surface area occupied by the thyroid (μm^2 = microns squared), while volume is the amount of space encompassed by the cup-shaped pit of the evaginated portion of the thyroid (μm^3 = microns cubed).

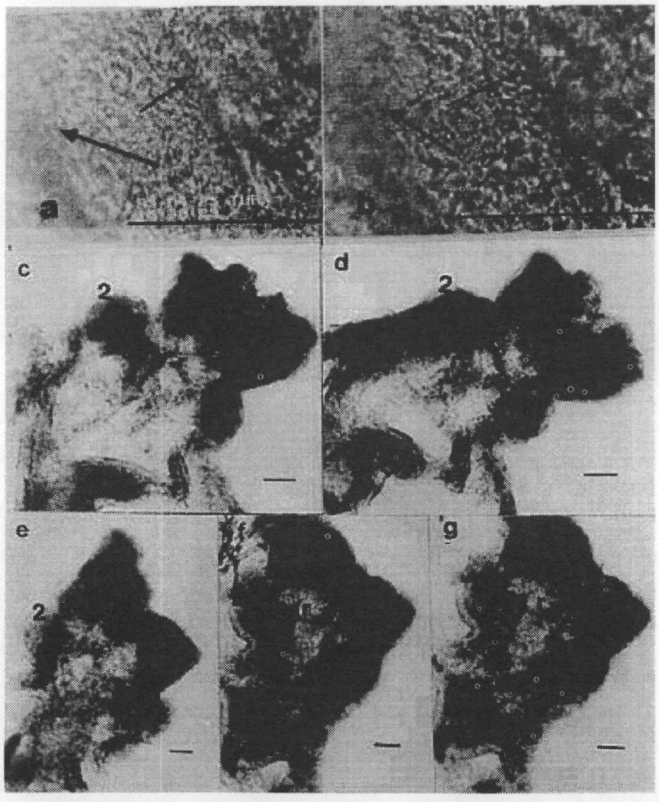

Figure 3. Treatment of chick thyroids with dihydrocytochalasin B. a,b) Thyroids of stage 12 embryos where a is untreated, while b was treated with 25 µM dihydrocytochalasin B in PBS for 20 minutes. Treatment did not result in any overt change in thyroid morphology. c,d) Pharynxes from stage 14 embryos where c is untreated and d was treated with 0.01 units/ml streptolysin O for 20 minutes. Treatment caused no obvious change in thyroid morphology. e,f,g) Pharynxes from embryos of stage 14- showing the effects of treatment with 15 µg/ml dihydrocytochalasin B-streptolysin O solution (CB+SLO). e) Untreated pharynx. f) Pharynx after 10 minutes of exposure to CB+SLO. g) Pharynx after 20 minutes of exposure to CB+SLO. Treatment caused the raised ridge of cells at thyroid margin to collapse. r = cranial region of the raised ridge of cells that surround the thyroid; 2 = second pharyngeal arch; bar = 45 µm.

TABLE 2

Treatment of thyroid with sihydrocytochalasin B with and without streptolysin O.

Treatment: [CB] * (µg/ml)	+ [SLO] • (units/ml)	Embryo♦ Stage	Effects◊
4.8	0	13+	none
10	0	17	none
12	0	12	none
15	0	17	none
20	0	15	none
25	0	14-	none
23.1	0.1	14-	collapse
0	0.1	14+	none
0	0.01	14;14	none
10	0.01	18	none
15	0.01	17;18	collapse
15	0.01	14;16	collapse
20	0.01	14;17	collapse
25	0.01	15	collapse

*[CB] = dihydrocytochalasin B in PBS.
•[SLO] = streptolysin O in PBS.
♦Entries under Embryo Stage show the stage of each embryo treated in the category.
◊collapse = peripheral cell ridge flattens, none = no morphological change observed.

constant while thyroid volume increases (Fig. 4). The increase in volume results from evagination of the thyroid primordium. These data support the assertion of Hilfer (1973) that lateral expansion of the thyroid into the surrounding pharyngeal space is somehow limited.

During the period of development encompassed by the present study, the floor of the pharynx and the thyroid primordium are one cell layer thick. As cells at the boundary between thyroid and pharynx convert from pharyngeal to thyroid tissue, they elongate to become spindle shaped, their apical microfilament bands thicken, and they become raised above the level of the adjacent pharyngeal cells to form a ridge (Shain and others 1972; Hilfer 1973). The population of cells that lie peripheral to the ridge is a mixture of pharyngeal cells and cells with some characteristics of thyroid cells.

Hilfer (1973) has suggested that the elevation of ridge cells is likely associated with thickening of their apical microfilament bands. His suggestion is supported by our observation that treatment of developing pharynxes with dihydrocytochalasin B causes this ridge to flatten and spread (Fig. 3). Additional support for this hypothesis comes from the observation that the cells most affected by exposure to ATP-containing medium were those corresponding to the region of cells that will form the ridge. Treatment of embryonic chick pharynxes with ATP-containing medium caused precocious evagination of thyroid primordia which included the elevation of cells that form the thyroid-pharyngeal border (Hilfer and others 1977).

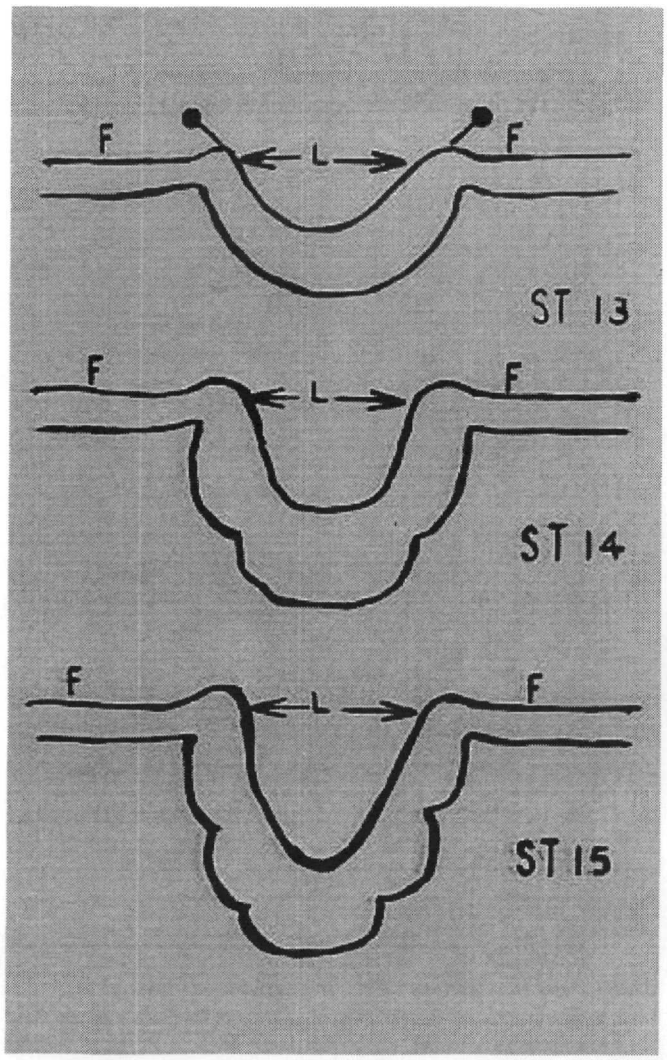

FIGURE 4. Diagrammatic cross-sections through the chick thyroid. The sketches show the changes in depth that occur in the thyroid during the period from stage 13 to stage 15. No significant changes occur in the dimension "L" during the same period. L = the lateral dimension used to calculate area (see Fig. 1), ST = developmental stage, F = pharyngeal floor, ↖ = indicates position of elevated ridge.

In addition to the forces exerted by microfilament contraction, it is likely that formation of the thyroid ridge is influenced by forces generated in the adjacent tissue. Precocious ridge formation failed to occur when the developing thyroid was excised from the pharyngeal floor and no ridge formed in regions of the pharynx where slits were made along the edge of the thyroid (Hilfer and others 1977). Odell and others (1981) suggest that microfilaments can be stimulated to contract by stretch forces acting on the cell. It is possible that cells of the pharyngeal floor exert such forces on adjacent thyroid cells, most likely in the form of shear stresses caused by the higher rate of mitotic activity in the pharyngeal cell population (Smuts and others 1978). Such forces would stimulate contraction of subapical microfilaments in the peripheral thyroid cells resulting in elongation of the cells and the subsequent elevation of the ridge. According to the model of Odell and others (1981), once contracted, the microfilaments would stabilize the new morphological configuration of the thyroid cells.

Alternatively, compressive forces generated by the more mitotically active pharyngeal population could result in elevation of the immediately adjacent thyroid cells in a manner similar to that proposed for caudal neural fold elevation in the chick (Schoenwolf and Smith 1990). In this case, thickening and contraction of microfilaments of the thyroid ridge cells may be differentiation events and serve only to stabilize the ridge. However, several observations argue for the former rather than the latter explanation: 1) elevation of the ridge can be caused to occur precociously in response to a contraction-causing medium; 2) the reported histological changes associated with precocious evagination resemble those that are expected to result from microfilament contraction (Hilfer and others 1977); and 3) the ridge is lost on exposure to microfilament disrupting treatment (Fig. 3).

The present study clearly demonstrates that at the same time lateral expansion of the developing thyroid is limited, growth and evagination of the anlage is occurring. The increase in thyroid volume could occur by one or more of the following mechanisms: 1) mitosis of thyroid cells, 2) elongation of thyroid cells, or 3) annexation of adjacent pharyngeal cells into the thyroid primordium. Previous work by Smuts and others (1978) has suggested that the rate of mitosis in the developing thyroid is lower than that in adjacent pharyngeal tissue. Recent work in our laboratory (Blayney 1999) suggests that expression of the epidermal growth factor receptor (EGFR) is depressed in developing thyroid cells relative to the level of expression in developing pharyngeal cells. Since epidermal growth factor is a known mitogen for various mesodermal and epidermal tissues, and since this factor exerts its mitogenic effect via binding to its receptor, the finding of depressed levels of EGFR in thyroid tissue relative to pharyngeal tissue is consistent with the suggestion that developing thyroid cells divide more slowly than surrounding pharyngeal cells. This finding implies, in turn, that annexation of cells from the pharynx into the developing thyroid and/or the elongation of thyroid cells is responsible for the increase in thyroid volume reported in the present study. Studies investigating the regulation of mitosis in the developing thyroid are currently underway in our laboratory.

ACKNOWLEDGMENTS. We are grateful for the patience and time Dr. Valerie Flechtner gave to this project as a mentor and reader. Thanks also to Dr. Miles Coburn whose final critique was invaluable. Support for this project was provided by a John Carroll University Graduate Assistantship and Faculty Research Grant.

LITERATURE CITED

Bhakdi S, Roth M, Sziegoleit A, Tranum-Jensen J. 1984. Isolation and identification of two hemolytic forms of streptolysin-O. Infect Immun 46(2):394-400.

Blayney SL. 1999. The involvement of epidermal growth factor receptors in thyroid outpocketing. [MSc thesis], John Carroll University.

Bonder EM, Mooseker MS. 1986. Cytochalasin B slows but does not prevent monomer addition at the barbed end of the actin filament. J. Cell Biol. 102:282-8.

Burnside B. 1973. Microtubules and microfilaments in amphibian neurulation. Am Zool 13:989-1006.

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88(1):49-92.

Hardin J. 1990. Context-sensitive cell behaviors during gastrulation. Seminars in Dev Biol 1:335-45.
Hilfer SR. 1973. Extracellular and intracellular correlates of organ initiation in the embryonic chick thyroid. Am Zool 13:1023-38.
Hilfer SR, Palmatier BY, Fithian EM. 1977. Precocious evagination of the embryonic chick thyroid in ATP-containing medium. J Embryol exp Morph 42:163-75.
Hopkins ML. 1935. Development of the thyroid gland in the chick embryo. J Morphol 88:49-92.
Hugo F, Reichwein J, Arvand M, Kramer S, Bhakdi S. 1986. Use of a monoclonal antibody to determine the mode of transmembrane pore formation by streptolysin O. Infect Immun 54(3):641-5.
Koehl MAR. 1990. Biomechanical approaches to morphogenesis. Seminars in Dev Biol 1:367-78.
Lewis WH. 1955. Mechanics of invagination. Anat Rec 97:139-56.
Mabuchi I. 1983. Electron microscopic determination of the actin filament end at which cytochalasin B blocks monomer addition using the acrosomal actin bundle from horseshoe crab sperm. J Biochem (Tokyo) 94:1349-52.
Odell GM, Oster G, Alberch P, Burnside B. 1981. The mechanical basis of morphogenesis: Epithelial folding and invagination. Dev Biol 85:446-62.
Schoenwolf GC, Smith JL. 1990. Mechanisms of neurulation: traditional viewpoint and recent advances. Development 109:243-70.
Schroeder TE. 1973. Cell constriction: contractile role of microfilaments in division and development. Am Zool 13:949-60.
Shain WG, Hilfer SR, Fonte VG. 1972. Early organogenesis of the embryonic chick thyroid. 1. Morphology and biochemistry. Dev Biol 28:202-18.
Smuts MS, Hilfer SR, Searls RL. 1978. Patterns of cellular proliferation during thyroid organogenesis. J Embryol exp Morph 48:269-86.
Wessells NK, Spooner BS, Ash JF, Bradley MO, Luduena MA, Taylor EL, Wrenn JT, Yamada KM. 1971. Microfilaments in cellular and developmental processes. Science 171:135-43.